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Regulation of SIRT1 protein levels by nutrient availability

Yariv Kanfi, Victoria Peshti, Yosi M. Gozlan, Moran Rathaus, Reuven Gil, Haim Y. Cohen*

The Mina & Everard Goodman Faculty of Life Sciences, Bar-Ilan University, Ramat-Gan 52900, Israel

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Abstract The mammalian NAD+ dependent deacetylase, SIRT1, was shown to be a key protein in regulating glucose homeostasis, and was implicated in the response to calorie restriction. We show here that levels of SIRT1 increased in response to nutrient deprivation in cultured cells, and in multiple tissues of mice after fasting. The increase in SIRT1 levels was due to stabilization of SIRT1 protein, and not an increase in SIRT1 mRNA. In addition, p53 negatively regulated SIRT1 levels under normal growth conditions and is also required for the elevation of SIRT1 under limited nutrient conditions. These results have important implications on the relationship between sirtuins, nutrient availability and aging.

Keywords: SIRT1; Sirtuins; Nutrient availability

1. Introduction

The sirtuins are a family of proteins homologous to the *Saccharomyces cerevisiae* NAD+ dependent protein deacetylase, ySir2. In yeast, members of the family were shown to be involved in transcriptional silencing, telomere maintenance and the regulation of life span. Mutation in ySir2 shortens the yeast replicative life span by 40%, whereas increasing the activity of ySir2, using either genetic [1] or chemical means [2], extends yeast life span by a similar extent. Similarly, over expression of *Caenorhabditis elegans* [3] and *Drosophila melanogaster* [4] sirtuins extends the life spans of these organisms by 30–40%. In addition, mutations in ySir2 or Drosophila Sir2 block the beneficial effect of a calorie restricted (CR) diet on life span [5]. The latter observation suggests that sirtuins are required for mediating the increase in life span induced by limited nutrient supply. CR slows the rate of aging and extends the maximum life span of a wide range of organisms, including yeast, nematodes, drosophila and rodents [6,7]. Moreover, CR had been found to delay the appearance of many age-related disorders, such as cancer, declining immune function, diabetes and metabolic syndrome phenotypes [8]. However, to date, the molecular mechanism by which mammalian sirtuin homologues, known as SIRT1-7, might retard aging and how CR is regulated are poorly understood.

One possible mechanism for the involvement of sirtuins in regulating life span could be via their affect on metabolism, in particular the homeostasis of glucose and fat. To date, five mammalian sirtuins, SIRT1–4 and SIRT6, were shown to play a key role in regulating metabolism. Most of our knowledge on the involvement of sirtuins in metabolitic pathways is based on SIRT1. The regulation of glucose and fat metabolism by SIRT1 is complex. SIRT1 promotes fat mobilization in white adipose tissue by repressing PPARγ (peroxisome proliferator co-repressor)-mediated transcription [9]. Over expression of SIRT1 or activation of its activity by resveratrol promotes lipolysis and fat loss in differentiated fat cells [9]. Similarly to SIRT1, SIRT2 negatively controls adipogenesis by modulating FOXO1 acetylation and phosphorylation thereby inhibiting its activity [10]. SIRT1 also positively regulates the liver X receptor (LXR), which functions as a cholesterol sensor and controls whole body cholesterol and lipid homeostasis [11].

SIRT1 regulates glucose homeostasis by controlling insulin secretion in response to glucose and insulin sensitivity. A series of in vivo experiments demonstrated that SIRT1 positively regulates insulin secretion in pancreatic β cells [12,13] by suppressing the transcription of uncoupling protein 2 (UCP2), a negative regulator of insulin secretion [12]. SIRT1 also augments insulin sensitivity by repressing the transcription of protein tyrosine phosphatase1 – PTP1B and enhancing phosphorylation of IRS-2, a negative regulator and a vital step in the insulin transduction, respectively [14,15]. Whether SIRT1 regulates insulin sensitivity through other pathways is not yet known, as contradictory reports were published on the role of SIRT1 in regulating adiponectin [16,17], an adipose-derived hormone whose secretion sensitizes the liver and the muscles to insulin.

Sirtuins were shown to be regulated by both nutrient levels and CR. SIRT1 protein levels are elevated in rats maintained on a CR diet [18], and SIRT1 transcription is induced upon nutrient deprivation *in vitro* in a p53-dependent manner [19]. Caloric restriction also activates SIRT2 [20] and SIRT3 [21] expression in adipose tissues, but represses SIRT4 [22]. Recently it was demonstrated that SIRT6, the only known sirtuin whose absence in mice causes premature appearance of aging-related pathologies, also increases in CR rats, in mice after 24 h of fasting and in cell cultures after nutrient depletion [24]. Interestingly, the increase in SIRT6 level is due to an increase in its protein stability, and not enhanced transcription.

Here, we examined the mechanism by which SIRT1 increases upon nutrient depletion, in particular whether it is controlled by elevated transcription, or is (also) regulated by increased protein stability, and whether the effect is tissue specific or not. To this end, we measured SIRT1 protein levels in mice after 24 hours of fasting and in cells growing in the absence of serum and glucose. We show here that SIRT1 levels increase in multiple mouse tissues after 24 hours of fasting,

*Corresponding author. Fax: +972 3 738 4058.
E-mail address: cohenh6@mail.biu.ac.il (H.Y. Cohen).

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and in cells growing in the absence of serum and glucose; SIRT1 levels are more strongly influenced by the absence of serum than by the absence of glucose; the affect on SIRT1 level was in a threshold manner of regulation; the increase in SIRT1 upon nutrient deprivation is mostly due to an increase in its protein stability, and p53 is a negative regulator of SIRT1 expression. Thus, taken together, these data suggest the involvement of SIRT1 in the response to nutrient levels and that increased protein stability rather than transcription is the mechanism for elevated SIRT1 levels upon nutrient depletion. Together these findings raise the possibility that part of the influence of CR on age-related metabolic disorders is mediated by increases in the SIRT1 level.

2. Results

SIRT1 was shown to be a regulator of metabolism [23]. To understand if nutrient levels directly regulate the level of SIRT1 protein, SIRT1 protein levels were monitored in mammalian models of nutrient deprivation. In human embryonic kidney 293 fibroblasts (HEK 293) and rat pheochromocytoma PC12 cells, the level of SIRT1 was elevated after nutrient deprivation induced by the absence of serum and glucose (Fig. 1A). These findings suggest that SIRT1 protein levels are regulated in vitro by nutrient deprivation.

To determine whether the SIRT1 response to nutrient levels was dose-dependent or represented a threshold response, HEK293 cells were grown in glucose-free growth medium, with decreased concentrations of serum, or in serum-free growth medium with decreased concentrations of glucose. As seen in Fig. 1B, SIRT1 levels were more sensitive to the absence of serum than glucose. Quantitative measurement of the changes in SIRT1 upon glucose or serum starvation showed that the response to serum or glucose levels was in a threshold manner (Fig. 1C). Interestingly, the absence of glucose makes the cells predisposed to the absence of serum.

To address the mechanism by which SIRT1 levels increased, we examined its levels in HEK293 cells under nutrient-deprived conditions in the presence and absence of various inhibitors. Treatment with the transcription inhibitor, actinomycin D, for the last 4 h or the entire 24-h starvation period, reduced the starvation-dependent increase in SIRT1 levels by at least 65% (Fig. 2A). Treatment with the translational inhibitor, cyclohexamide, had a similar effect on SIRT1 levels under starved conditions. Yet, no change in SIRT1 mRNA was detected in nutrient-deprived HEK293 cells by RT-PCR analyses (Fig. 2B and Suppl. Fig. 1). Recently, we demonstrated that the increase in SIRT1 following nutrient deprivation was not due to changes in the proteasomal degradation of SIRT1 [24]. Taken together, these findings suggest that nutrients depletion does not affect SIRT1 protein levels via its transcription. Thus, at least in HEK293 culture, the increase in SIRT1 protein is mediated by another, as yet unidentified, protein as treatment with actinomycin D affected SIRT1 levels.

Previous reports in which the levels of SIRT1 were followed in vivo in starved mice examined SIRT1 levels in a limited number of tissues, and at the RNA level only [19]. Yet, it is possible that the reported observed increase in the RNA levels of SIRT1 in these experiments did not necessarily represent a

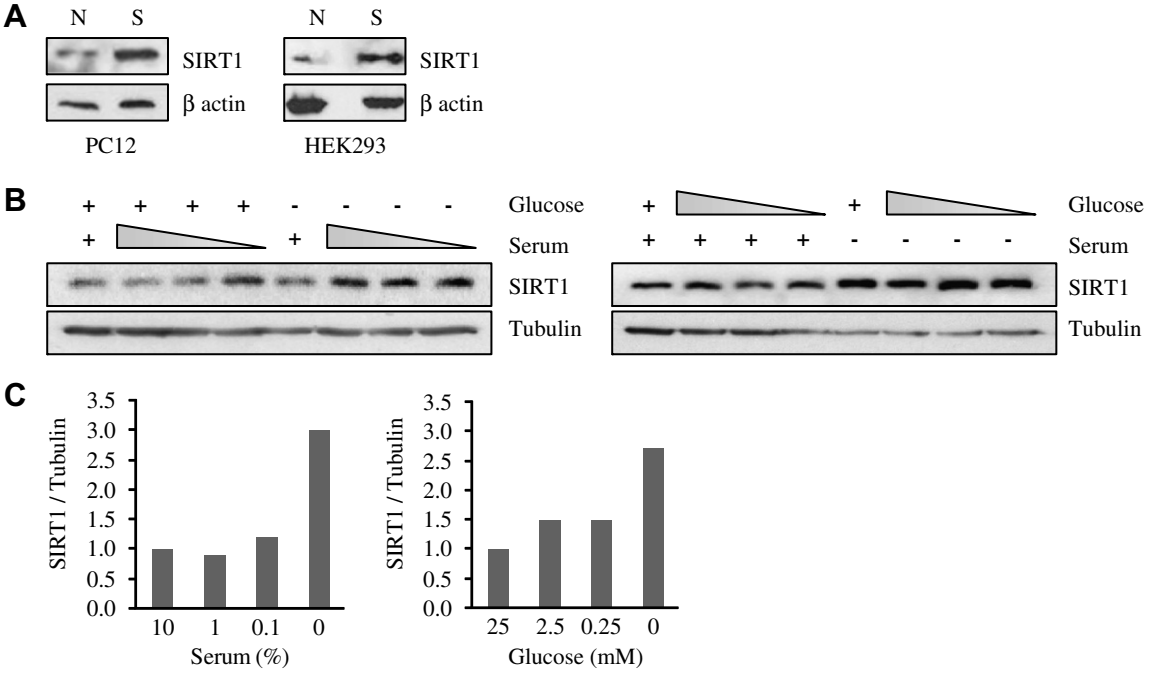

Fig. 1. SIRT1 protein levels increase upon nutrient deprivation. (A) SIRT1 protein levels in rat pheochromocytoma PC12 cells or human embryonic kidney HEK293 cells grown in complete– [N], or starvation (without serum and glucose) – [S] medium. (B) HEK293 cells were grown in decreasing ten fold dilutions of serum (left panel) or glucose (right panel). Cells grown in complete medium served as a control in each experiment (left lane). β-Actin or tubulin served as loading controls. (C) Graphic representation of the changes in SIRT1 levels in dependent of serum (left) or glucose (right) concentration. Each graph represents the first four lanes of the corresponding analysis in B. Band intensity measurements were done using ImageJ analysis.

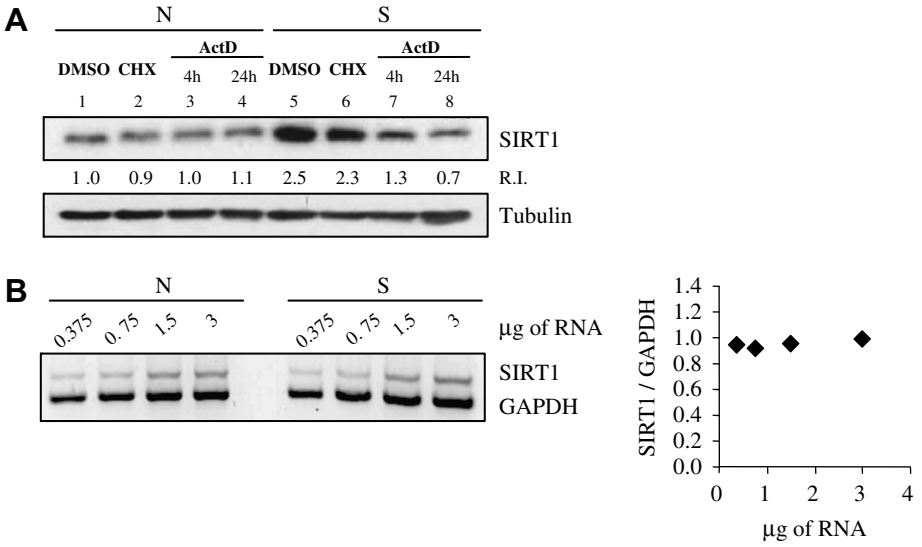

Fig. 2. SIRT1 transcription is unchanged upon nutrient deprivation. (A) SDS–PAGE analysis of SIRT1 in HEK293 cells supplemented with normal levels of nutrients – [N] or starved – [S] in the presence and absence of the following agents: The translation inhibitor cyclohexamide (CHX) added for 4 h, the transcriptional inhibitor, actinomycin D (ActD), added for 4 or 24 h. Cells were grown under either normal or starvation conditions in the presence of dimethyl sulfoxide (DMSO), which served as a solvent control. Tubulin was used as a loading control. In each panel, the relative intensity [RI] of a given band relative to the loading control (tubulin) is indicated below each growth condition. (B) Semi quantitative RT-PCR analysis of SIRT1 gene transcription in HEK293 cells under normal – [N] or starvation – [S] conditions. RNA templates were serially diluted (dilution of the RNA is indicated above the panel). The ratio between SIRT1 mRNA and GAPDH mRNA in each sample, as measured by densitometric analysis, was plotted and is shown in a graph (right panel). Band intensity measurements were done using ImageJ analysis.

change in SIRT1 protein levels. Thus, we surveyed SIRT1 levels in mice fed an ad libitum (AL) diet and in mice after 24 h of fasting (supplemented with water only). SIRT1 protein was assessed in the following tissues: brain, spleen, muscle, heart, kidney, liver, white adipose tissue (WAT) and testis. After 24 h of fasting, SIRT1 protein levels were elevated in brain, heart, muscle, WAT and kidney (Fig. 3A), but not in the other tissues tested. Interestingly as observed in tissue culture, RT-PCR showed that in vivo SIRT1 mRNA did not increase in the brain, kidney or muscle after nutrient deprivation, despite increased protein levels (Fig. 3B). Real-time PCR also revealed that SIRT1 mRNA levels in the muscle did not increase upon starvation (Fig. 3C).

The tumor suppressor p53 was shown to regulate SIRT1 RNA levels in mice fed a normal diet, and to induce the increase in the RNA levels upon starvation [19]. As described above, it is possible that SIRT1 is strictly regulated at the protein level, and those changes in RNA levels are not manifest at the level of protein expression. Thus, we tested whether the protein levels of SIRT1 might be regulated by p53. To this end, SIRT1 protein levels were evaluated in brain and heart tissues of wild-type or p53-deficient ($p53^{-/-}$) mice, and were found to be higher in the absence of p53 (Fig. 4A). Thus, p53 regulates SIRT1 under normal growth conditions of AL diet. In agreement with Nemoto et al. [19], the increase in SIRT1 levels was in part due to elevated transcription as quantitative real-time PCR show a significant increase in SIRT1 mRNA in the brain of $p53^{-/-}$ mice (Fig. 4B). Interestingly, in p53-deficient mice, SIRT1 protein or RNA levels did not significantly increase after 24 h of fasting (Fig. 4C and D). To address the mechanism by which p53 affects SIRT1 levels, we examined its levels in HEK293 cells under nutrient-deprived conditions in the presence and absence of various inhibitors. Upon starvation the level of p53 increases by 40%. Treatment with the transcription inhibitor, actinomycin D, for the last 4 h of the entire 24-h starvation period, reduced the starvation-dependent increase in p53 levels by at least 40% (Fig. 4E). Treatment with the translational inhibitor, cyclohexamide, reduces p53 levels only in AL diet. Taken together, our results demonstrate that p53 stabilized in starved HEK293 cells and p53 is a negative regulator of SIRT1 protein levels under normal growth conditions. Yet, its presence is necessary for the increase in SIRT1 levels under conditions of nutrient limitation.

3. Discussion

Observations in yeast and flies suggest that sirtuin levels might be regulated by nutrient availability [23]. Previous studies demonstrated that SIRT1 protein levels increase in rats after 12 months of CR diet [18]. Similarly, other reports showed that SIRT1 transcription levels increase in fasting mice or in cells grown in the absence of serum and glucose [19]. Yet, many open questions remain. For example, it was not clear if SIRT1 protein levels also increase in fasting mice. Moreover, studies of other sirtuins showed that the increase in SIRT6 levels upon nutrient deprivation is regulated mainly on the protein level and not by transcriptional control [24]. Thus, we wished to explore whether SIRT1 is also controlled under a similar mode of regulation. In addition, it was not clear whether the degree of increase of SIRT1 in nutrient depleted cultured cells is dependent on the extent of nutrient deprivation, and whether it is triggered by the absence of serum, glucose or both. Here we show that (1) elevated SIRT1 levels are induced by 24 h of nutrient depletion in vitro and in vivo; (2) SIRT1 induction is tissue specific, being most pronounced in the brain, heart, muscle, kidney and white adipose tissues;

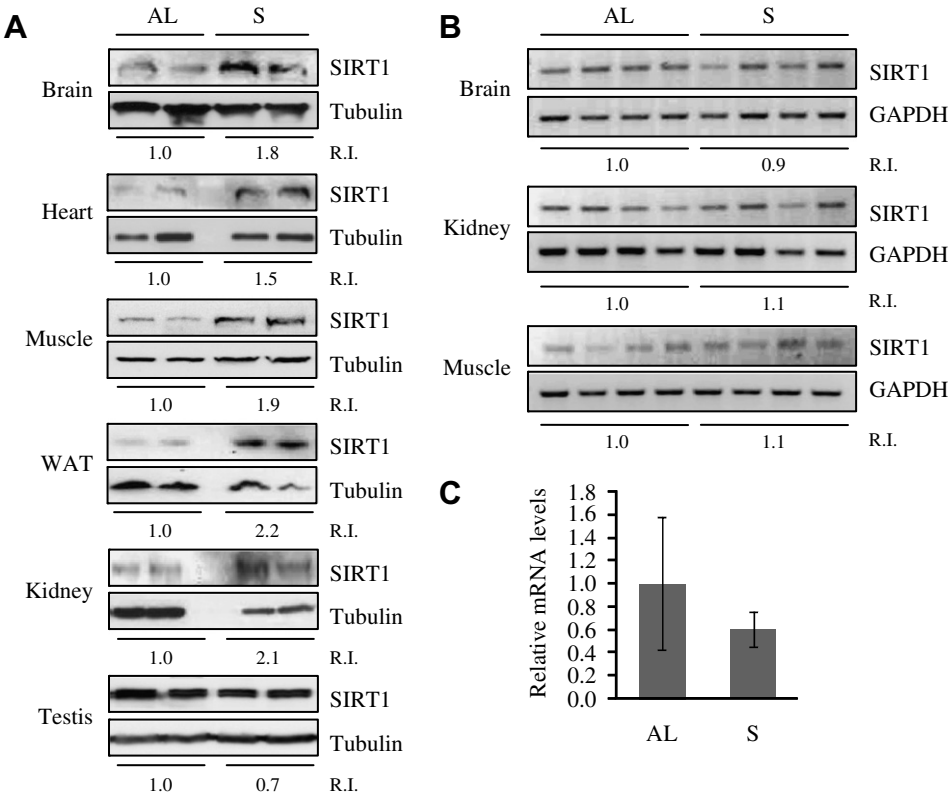

Fig. 3. SIRT1 protein but not RNA levels increase in multiple mouse tissues upon starvation. (A) Extracts of brain, heart, muscle, white adipose (WAT), kidney or testis tissues from mice fed an ad libitum diet – [AL], or mice fed a water only diet for 24 h – [S] were separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and probed with a specific rabbit polyclonal antibody against SIRT1. The data generated from two (out of four) representative mice from each treatment are shown; each lane represents an individual mouse. In each panel, the relative intensity [RI] of a given band relative to the loading control (tubulin) is indicated below each growth condition. Band intensity measurements were performed using ImageJ analysis. (B) Semi quantitative RT-PCR analysis of SIRT1 gene transcription of brain, kidney and muscle tissues from mice fed an ad libitum diet – [AL], or mice fed a water only diet for 24 h – [S]; each lane represents an individual mouse. In each panel, the relative intensity [RI] of a given band relative to the loading control (GAPDH) is indicated below each growth condition. The identity of each sample in parts A and B is detailed in Supp. Table 1. (C) Quantitative real-time PCR analysis of SIRT1 mRNA levels in the muscle tissues from mice fed an ad libitum diet – [AL], or mice fed a water only diet for 24 h – [S]. The graph show the average expression levels of four mice for each treatment. TATA-box binding protein (TBP) served as control to normalize expression.

(3) elevated SIRT1 levels after nutrient deprivation are not due to increased transcription; (4) SIRT1 levels are sensitive to the absence of serum or glucose depletion in the growth media in a threshold manner of regulation and (5) p53 negatively regulates SIRT1 protein levels. Taken together, these results suggest that SIRT1 protein levels are regulated by nutrient intake, and that one way by which nutrient limitation acts to extend life span is by increasing SIRT1 levels. This increase has the potential to increase SIRT1 activity, supporting glucose and fat homeostasis.

Similar to SIRT1, SIRT6 levels also increase upon nutrient deprivation. This similarity suggests that multiple sirtuins are subject to regulation by nutrient levels and CR diet. If so, how broad is the affect of CR on sirtuin levels? Limited knowledge exists regarding the regulation of other sirtuins by nutrient levels (Table 1). For example, it was shown that in CR mice the level of SIRT2 protein increases in the brain [20], and that SIRT3 RNA levels increase in their brown adipose tissue [21]. Interestingly, SIRT4 protein levels decrease in liver of CR mice [22]. This last observation adds another dimension of complexity, showing that nutrient limitation does not always increase sirtuin levels. Based on our current and previous data, both SIRT1 and SIRT6 [24] are regulated at the protein level upon nutrient deprivation. Such regulation might allow the organism to more quickly adapt to changes in nutrient level or to immediate stresses. How similar is the mode of regulation on SIRT1 and SIRT6 protein levels is still open. One major different is that whereas SIRT6 levels are regulated by proteasomal degradation those of SIRT1 does not [24]. In addition, in long term CR diet, which differs from starvation in some of its physiological effects, the increase in SIRT1 will affect SIRT1 regulated pathways including adipogenesis and insulin secretion [9,12,13]. Thus, given this similarity between SIRT1 and SIRT6 it would be of great interest to follow the involvement of SIRT6 in these pathways.

In mice fed an ad libitum diet p53 is a negative regulator of SIRT1 levels (Fig. 4A and B). Interestingly, SIRT1 deacetylates p53 in vivo [25] and in vitro [26], which potentially inhibits p53 activity and reduces its stability. These finding suggest a feedback regulation between p53 and SIRT1. It would be of great interest to follow this relationship under conditions that require the activity of p53. For example, under starvation p53 levels increase (Fig. 4E and [27]) and was also required for the increase in SIRT1 levels (Fig. 4C and D). We are currently

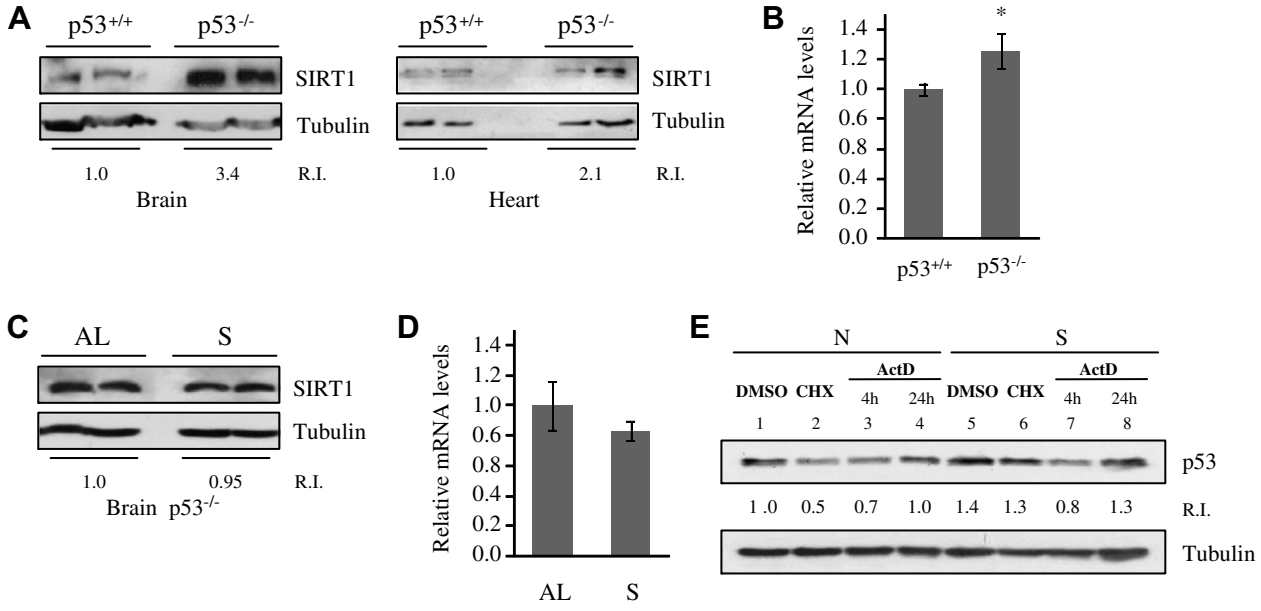

Fig. 4. p53 regulates SIRT1 levels. (A) Protein extracts of brain or heart tissues from wild-type or $p53^{-/-}$ mice maintained on a normal diet were separated by SDS–PAGE, and SIRT1 protein levels were measured with specific rabbit polyclonal antibody. (B) Quantitative real-time PCR analysis of SIRT1 mRNA levels in the brain tissues from $p53^{+/+}$ and $p53^{-/-}$ mice. SIRT1 protein levels (C) and quantitative real-time PCR analysis of SIRT1 mRNA levels (D) in the brain were also measured in $p53^{-/-}$ mice fed normally [AL], or with water only for 24 h – [S]. (E) SDS–PAGE analysis of p53 in HEK293 cells supplemented with normal levels of nutrients – [N], or starved – [S] in the presence and absence of the following agents: The translation inhibitor cyclohexamide (CHX) added for 4 h, the transcriptional inhibitor, actinomycin D (ActD), added for 4 or 24 h. Cells were grown under either normal or starvation conditions in the presence of dimethyl sulfoxide (DMSO), which served as a solvent control. In each panel, the relative intensity [RI] of a given band relative to the loading control (tubulin) is indicated below the growth conditions. Band intensity measurements were performed using ImageJ analysis. Asterisk – $P = 0.027$.

Table 1
Regulation of sirtuins by nutrient deprivation

Sirtuins	Human[a]		Rat		Mouse		Mechanism
	RNA	Protein	RNA	Protein	RNA	Protein	
SIRT1	√ [19]	√ [18,19,24][b]	ND	√ [18][b]	√ [19]	√ [24][b]	Protein stability contradictory report suggest an increase in transcription [19][b]
SIRT2	ND	ND	ND	ND	ND	ND [20]	ND
SIRT3	ND	ND	ND	ND	ND [21]	ND	ND
SIRT4	ND	ND	ND	ND	ND	ND [22]	ND
SIRT5	ND	ND	ND	ND	ND	ND	ND
SIRT6	√ [24]	√ [24]		√ [24]	√ [24]	√ [24]	Increased protein stability [24]
SIRT7	ND	ND	ND	ND	ND	ND	ND

Summary of the current state of knowledge on the regulation of sirtuins by nutrient availability. In all cases besides SIRT4 in liver of CR mice, nutrient limitation results in an increase in the sirtuin levels. RNA – shown by RT-PCR analysis, protein – shown by Western-blotting analysis and ND – no results have been published.
[a]Tissue culture only.
[b]Current study.

investigating whether the reciprocal inhibition between SIRT1 and p53 change under these conditions.

Both SIRT1 and SIRT6 were implicated in aging, and their levels increase upon nutrient depletion. In addition both are regulated by p53, which was shown to affect the regulation of aging of several organisms [28]. Yet, the opposite regulatory effect of p53 on SIRT1 and SIRT6 is puzzling. In vivo, p53 positively affects SIRT6 levels and negatively regulates SIRT1 levels. Thus, this opposite regulation might indicate that SIRT1 and SIRT6 oppositely mediate p53-related pathways including stress responses, apoptosis or senescence. It would be of a great interest to identify such pathways. For example, it was recently shown that SIRT6 is required for telomere maintenance, and in the absence of SIRT6, cells enter senescence earlier than wild-type cells [29]. In contrast, cells deficient in SIRT1 senesce significantly later than wild-type cells [30]. However, other reports demonstrated that the inhibition of SIRT1 by Sirtinol results in a decrease rather than an increase in senescence [31]. Thus, solving the triangle of p53, SIRT1 and SIRT6 is one of the major challenges in the field of aging research.

4. Experimental procedures

4.1. Cell culture

All cell lines were maintained as previously described [24]. For in vitro nutrient starvation, the cells were grown without serum and glucose for 24 h.

4.2. Rodents

Male C57BL mice were either fed AL, or given only water for 24 h (starvation) prior to analysis. Protein extraction from the tissues was done as described previously [24]. All experiments were approved by the Institutional Animal Care and Use Committee.

4.3. Antibodies and Western blot

Whole cell extracts were prepared using lysis buffer (50 mM Tris [pH 8], 1% NP-40, 150 mM NaCl, 1 mM MgCl2, 10% glycerol, 1 mM DTT and 1X EDTA-free protease inhibitor cocktail [Roche Diagnostics]). The following antibodies were used: mouse monoclonal anti-β-actin (A4700 Sigma), mouse monoclonal anti-α-tubulin (12G10 Hybridoma Bank, University of Iowa), rabbit polyclonal anti-SIRT1 (07-131 Millipore) and mouse monoclonal anti-p53 (DO-1 Santa Cruz).

4.4. RT-PCR

RT-PCR was performed as previously described [24].

4.5. Quantitative real-time PCR

RNA samples were extracted using RNeasy kit (QIAGEN) and quantified with NanoDrop 1000. The cDNA was synthesized using 0.3 mg total RNA and RT-PCR was performed using oligo dT primers (Revertaid, Fermentas). Quantitative real-time PCR was performed using 8 μl diluted cDNA/reaction using Absolute Blue QPCR Sybr-green Mix (Thermo) in a Chromo4 instrument cycler (Bio-Rad). TATA-box binding protein (TBP) was used as a control to normalize expression. For statistical analysis, each data point was determined in triplicate. The results were analyzed using the Genex Microsoft Excel plug-in program (Bio-Rad).

4.6. Primers

For RT-PCR analysis, the following SIRT1 specific primers were used:

Mouse SIRT1
S1 Fwd – 5′-TTGTGAAGCTGTTCGTGGAG-3′.
S1 Rev – 5′-GGCGTGGAGGTTTTTCAGTA-3′.
Human SIRT1 primers
Set #1:
S1 Fwd – 5′-CTGGACAATTCCAGCCATCT-3′.
S1 Rev – 5′-GGGTGGCAACTCTGACAAAT-3′.
Set #2:
S1.1 Fwd – 5′-GAACATAGACACGCTGGAAC-3′.
S1.1 Rev – 5′-GCTGCTTGGTCTAAAAGTGTG-3′.

For real-time PCR analysis, the following specific primers were used:

Mouse SIRT1
S1 Fwd – 5′-AGAACCACCAAAGCGGAAA-3′.
S1 Rev – 5′-TCCCACAGGAGACAGAAACC-3′.
TBP
T Fwd – 5′-ACCCTTCACCAATGACTCCTATG-3′.
T Rev – 5′-TGACTGCAGCAAATCGCTTGG-3′.

4.7. Inhibition of transcription or translation

For inhibition of transcription, the culture medium was supplemented with actinomycin D (5 μg/ml) for the entire 24 h of culture or for the last 4 h of the experiment. For inhibition of translation, the medium was supplemented with cyclohexamide (50 μg/ml) 4 h before harvesting the cells.

4.8. Statistical analysis

Statistical significance was evaluated by the two-tailed Student's *t-test* for unpaired data. A P value of less than 0.05 was considered statistically significant.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2008.06.005.

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